Distribution and function of GABA receptor ρ subunits in the rat nervous system

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ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I Wegelius K, Reeben M, Rivera C, Kaila K, Saarma M, Pasternack M. The ρ_1 GABA receptor cloned from rat retina is down-modulated by protons. *NeuroReport* 7, 2005-2009, 1996.

II Wegelius K, Pasternack M, Hiltunen JO, Rivera C, Kaila K, Saarma M, Reeben M. Distribution of GABA receptor ρ subunit transcripts in the rat brain. *Eur J Neurosci*, 10, 350-357, 1998.

III Rivera C, Wegelius K, Reeben M, Kaila K, Pasternack M. Different sensitivities of human and rat ρ_1 GABA receptors to extracellular pH. *Neuropharmacology*, 39(6):977-989, 2000.

IV Wegelius K, Alakuijala A, Schmidt M, Hirvela E, Saarma M, Paulin L, Pasternack M. Postnatal expression of GABA receptor ρ subunits in the rat nervous system. Manuscript.

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ABBREVIATIONS

3-APA	3-aminopropyl-phosphinic acid
3-APMPA	3-aminopropyl(methyl)-phosphinic acid
3-APS	3-aminopropane-sulfonic acid
aa	amino acid
AOS	accessory optic system
bp	base pair
CACA	*cis*-aminocrotonic acid
CAMP	*cis*-2-aminomethyl-cyclopropylcarboxylic acid
cDNA	complementary deoxyribo-nucleic acid
CNS	central nervous system
CTX	neocortex
DAVA	δ-aminovaleric acid
DRG	dorsal root ganglia
DTN	dorsal terminal nucleus
GABA	γ-aminobutyric acid
HC	hippocampus
I4AA	imidazole-4-acetic acid
INL	inner nuclear layer
kb	kilobase pairs
LGNd	dorsal lateral geniculate nucleus
LTN	lateral terminal nucleus
mRNA	messenger ribonucleic acid
MTN	medial terminal nucleus
NOT	pretectal nucleus of the optic tract (pretectum)
OPL	optic layer
P-4-S	piperidine-4-sulfonic acid
P	postnatal day
PCR	polymerase chain reaction
pEPSP	population excitatory postsynaptic potential
PiTX	picrotoxin
RET	retina
RT	reverse-transcription
SuC	superior colliculus
SC	spinal cord
SGL	superficial gray layer
TACA	*trans*-aminocrotonic acid
TAMP	*trans*-2-aminomethyl-cyclopropylcarboxylic acid
TBPS	t-butylbicyclophophoro-thionate
THIP	4,5,6,7-tetrahydroiso-xazolo-(5,4-c)pyridin-3-ol
TM	transmembrane
TPMPA	(1,2,5,6-tetrahydro-pyridine-4-yl)-methylphosphinic acid

1. INTRODUCTION

1.1. GABA, AN INHIBITORY TRANSMITTER

Several experiments done in the 1950's and 1960's suggested that g-aminobutyric acid (GABA) has an inhibitory effect in the invertebrate (Boistel and Fatt, 1958; Kuffler and Edwards, 1958; Takeuchi and Takeuchi; 1965; Kravitz, 1963; Otsuka *et al.*, 1966) and the mammalian nervous system (Hayashi and Nagai, 1956; Bazemore *et al.*, 1957; Krnjevic and Schwartz, 1967; Dreifuss *et al.*, 1969). Today we know that GABA is the major inhibitory transmitter in the nervous system from crustaceans to mammals, and is found in 20-50% of synapses (for reviews, see Sivilotti and Nistri, 1991; Freund and Buzsaki, 1996). Suppression of inhibition mediated by GABA has been shown to cause severe hyperexcitability and seizures (Schwartzkroin, 1983; Krnjevic, 1983; Alger, 1984), and has been suggested to be involved in the mechanisms of epilepsy (Dichter, 1989; Macdonald, 1989; Kapur and Lothman, 1989; see also Mody, 1998).

In the vertebrate nervous system, GABA activates receptors that have been traditionally classified into $GABA_A$ and $GABA_B$ receptors based on their structure and pharmacology (Hill and Bowery, 1981). However, during the last decade the growing knowledge on GABA receptors has brought into light evidence for a third class of GABA receptors, which are now commonly known as the $GABA_C$ receptors.

1.2. IONOTROPIC GABA RECEPTORS

1.2.1. $GABA_A$ RECEPTORS

$GABA_A$ receptors are ionotropic receptors directly activated by GABA. They belong to a superfamily of ligand-gated ionotropic receptors, including e.g. nicotinic acetylcholine receptors (Schofield *et al.*, 1987). $GABA_A$ receptors mediate fast responses to GABA by opening an integral anion channel that is permeable to chloride (Cl^-) and bicarbonate (HCO_3^-) ions (Bormann *et al.*, 1987; Kaila and Voipio, 1987). The postsynaptic inhibition in the target neuron is largely based on the increase in postsynaptic conductance that stabilizes the membrane potential close to its resting level and suppresses both spatial and temporal summation of excitatory postsynaptic potentials, which leads to a decrease in the probability of spike firing ("shunting inhibition"). In addition to this, various kinds of neurons have a mechanism that actively extrudes chloride (see e.g. Rivera *et al.*, 1999), which is required for the generation of hyperpolarizing inhibitory postsynaptic potential ("voltage inhibition") (for review, see Kaila, 1994). These "classical" GABA responses were originally identified by their sensitivity to the convulsant drug picrotoxin, that blocks the ion channel, and to the plant alkaloid bicuculline, that antagonizes the ligand-binding site (Nistri and Constanti, 1979). Muscimol has been used to activate $GABA_A$ receptors selectively

from $GABA_B$ receptors. In addition, the GABA-induced activation of most $GABA_A$ receptors can be modulated by benzodiazepines, barbiturates, neurosteroids and ethanol (for review, see Bormann, 1988; Kaila, 1994; MacDonald and Olsen, 1994; Sieghart, 1995; Barnard *et al.*, 1998).

1.2.1.1. Several subunit types form $GABA_A$ receptors

The $GABA_A$ receptors are presumably pentameric structures composed of a selection of subunits from seven classes (Fig. 1). Six isoforms of α, four β, three γ, and single δ, ε, θ and π subunits have been cloned and sequenced from the vertebrate nervous system during the last decade (Wisden and Seeburg, 1992; Barnard *et al.*, 1998; Bonnert *et al*., 1999). In addition, several alternatively spliced forms of $GABA_A$ receptor subunits have been identified (Whiting *et al.*, 1990; Bateson *et al.*, 1991; Kofuji *et al.*, 1991; Harvey *et al.*, 1994; Korpi *et al.*, 1994). The subunits share 60-80 % similarity on the amino acid level within a subunit class, and 30-40 % between classes. Each subunit consists of four putative membrane-spanning regions, a large N-terminal extracellular domain, a short domain in the C-terminal end, and a long intracellular loop between the third and the fourth transmembrane regions which contains putative phosphorylation sites (Olsen and Tobin, 1990; Burt and Kamatchi, 1991).

Studies with different subunit isoforms expressed in *Xenopus* oocytes have revealed that many subunits are able to form homooligomeric receptors, but display small currents and rather inefficient assembly (Blair *et al.*, 1988; Pritchett *et al.*, 1988; Shivers *et al.*, 1989; Verdoorn *et al.*, 1990; Joyce *et al.*, 1993). The combination of α and β subunits are however needed to form a functional GABA-gated channel that mimicks the pharmacology of native $GABA_A$ receptors (Levitan *et al.*, 1988; Moss *et al.*, 1990). In addition, the γ subunit seems to be necessary for full benzodiazepine-sensitive $GABA_A$ receptor function (Pritchett *et al.*, 1989). It has been suggested that the γ subunit can be replaced by the δ subunit to form $GABA_A$ receptors that are insensitive to benzodiazepines. Studies using *in situ* hybridization and immunohistochemistry techniques on rat brain sections also show the expression of at least one α, β and γ or δ subunit in different brain regions (Laurie *et al.*, 1992; Fritschy *et al.*, 1994). Combining results obtained from experiments so far, $GABA_A$ receptors in the CNS are most likely formed by combinations of α and β subunits with one (or more) γ, δ, ε or π subunit (Sieghart, 1995; Barnard *et al.*, 1998).

The sensitivity to several modulatory agents has been shown to vary with subunit composition, and the properties of the $GABA_A$ receptor channels vary as well. Single-channel recordings suggest that the application of GABA can cause at least four different conductance states in $GABA_A$ receptors (Hamill *et al.*, 1983; Bormann *et al.*, 1987). However, most receptor channels seem to open to a 27-30 pS main conductance level (Bormann, 1988; Macdonald and Twyman, 1992).

In vitro measurements of transiently expressed $GABA_A$ receptors have revealed sigmoidal GABA concentration response curves with a half-maximal concen-

tration of GABA between 10-100 μM, and Hill coefficients of about two suggesting that at least two molecules of GABA are needed to activate $GABA_A$ receptors (Sakmann *et al.*, 1983). *In vitro* studies with homooligomeric receptor channels suggest that up to five binding sites for GABA might be present on $GABA_A$ receptors (Sieghart, 1995). The pore diameter of $GABA_A$ receptors of spinal cord neurons (Bormann *et al.*, 1987) as well as those of $\alpha_1\beta_2\gamma_2L$ receptors expressed in *Xenopus* oocytes (Wotring *et al.*, 1999) have been estimated to be 0.56 nm.

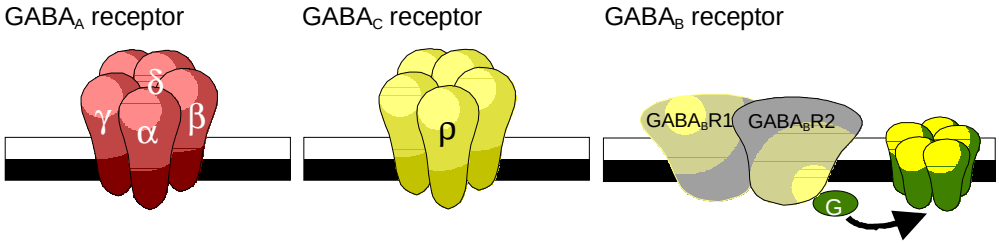

Fig. 1. Schematic drawing illustrating the molecular compositions of $GABA_A$, $GABA_B$ and $GABA_C$ receptors.

1.2.1.2. $GABA_A$ receptors are developmentally regulated

GABA is one of the earliest transmitters detected in the developing CNS and has been suggested to play an important role in the neuronal development (Lauder *et al.*, 1986; Ma *et al.*, 1991; 1995; Behar *et al.*, 1996; 1998). The $GABA_A$ subunits have been shown to exhibit unique regional and temporal developmental expression profiles (Laurie *et al.*, 1992; Fritschy *et al.*, 1994). In several brain regions, expression of some subunit mRNAs decreased during early postnatal development, while the expression of other subunits increased with age (Laurie *et al.*, 1992). In contrast, no developmental switch was seen in cerebellar Purkinje cells, where only few subunit types could be detected from birth to adult (Laurie *et al.*, 1992). These changes in structure or assembly of different subunits may be responsible for developmental modifications in function of the $GABA_A$ receptors as has been shown for recombinant $GABA_A$ receptors in *in vitro* expression systems (Sigel *et al.*, 1990; Verdoorn *et al.*, 1990).

In immature neurons, Cl^- extrusion mechanisms are apparently not fully developed, and GABA induces a depolarizing current acting mainly as an excitatory transmitter on ionotropic GABA receptors (Ben-Ari *et al.*, 1989; 1997; Cherubini *et al.*, 1991; Wall and Usowicz, 1997; Huang and Redburn, 1996). During the first postnatal week, in the pyramidal cells of the rat hippocampal CA3 region, spontaneous giant depolarizing potentials (GDPs) occur which are thought to be mediated by $GABA_A$ receptor channels (Ben-Ari *et al.*, 1989). The GDPs disappear at a time when the

reversal potential of the $GABA_A$ receptors shifts to hyperpolarizing (Ben-Ari *et al*., 1989). This developmental shift in the reversal potential is apparently caused by a K^+/Cl^- co-transporter KCC2 (Payne *et al*., 1996) that has been shown to extrude Cl^- from pyramidal cells after a developmental induction during early postnatal age (Rivera *et al.*, 1999).

1.2.1.3. Ionic modulation of $GABA_A$ receptors

Ionic shifts in the intra- and extracellular space have been found to have significant effects on $GABA_A$ receptors by producing changes in the efficacy of inhibition or by causing direct modulatory action on $GABA_A$ receptors (Traynelis, 1998). Especially certain cations, including protons (H^+), and zinc (Zn^{2+}), are known to modulate GABA receptors directly (Pasternack *et al.*, 1992; 1996; Westbrook and Mayer, 1987; Smart *et al.*, 1994; Traynelis, 1998).

Changes in pH and channel-mediated movements of acid-base equivalents have been suggested to have a modulatory effect on excitability in the brain. The modulatory effect of protons is well known (Somjen and Tombaugh, 1998), and is thought to be mediated through opposite pH sensitivities of ionotropic GABA and NMDA receptors. In several preparations, extracellular H^+ ions have been shown to enhance $GABA_A$-mediated responses (Gallagher *et al.*, 1983; Tang *et al.*, 1990), but in some studies the opposite effect has been detected (Gruol *et al.*, 1980; Smart, 1992; Huang and Dillon, 1999). In view of the H^+ sensitivity of the $GABA_A$ receptors, the possibility emerges that the channel-mediated movements of acid-base equivalents exert a modulatory action on the underlying inhibitory conductance mechanism (Kaila *et al.*, 1990). Changes in the intracellular pH seem to have little effect on $GABA_A$ receptors (Pasternack *et al.*, 1992), suggesting that the modulatory site(s) of H^+ lies in the extracellular domain of $GABA_A$ receptor subunits.

Zn^{2+} has been shown to be stored presynaptically in certain neurons of the CNS, and to be released during synaptic activity (Assaf and Chung, 1984; Frederickson and Danscher, 1990). In contrast to the effect of protons, it seems that Zn^{2+} inhibits both excitatory and inhibitory responses (Westbrook and Mayer, 1987; Traynelis and Cull-Candy, 1991; Smart and Constanti, 1990; Smart 1992), while in some neurons Zn^{2+} does not show any inhibiting effect (Smart and Constanti, 1983; 1990). Changes in Zn^{2+} sensitivity of $GABA_A$ receptors are seen during development, probably due to developmental differences in the subunit composition of the receptors (Smart, 1992, Draguhn *et al.*, 1990).

1.2.2. $GABA_C$ RECEPTORS

1.2.2.1. Evidence for an ionotropic GABA receptor separate from $GABA_A$ receptor

The first ones to propose the existence of GABA-activated anion channels distinct from $GABA_A$ receptors were Johnston and co-workers (Johnston *et al.*, 1975) who discovered GABA-activated responses that were not inhibited by the $GABA_A$ receptor antagonist bicuculline in cat spinal cord interneurons. They studied the effect of a conformationally restricted GABA analogue, *cis*-4-aminocrotonic acid (CACA) that was found to be ineffective on $GABA_A$ receptors, but had a depressant action on spinal interneurons, which could not be blocked by bicuculline, or strychnine, a glycine receptor antagonist.

A novel effect of GABA on neurotransmission was also reported in the central visual pathways. In the frog optic tectum, GABA was found to increase the optic nerve-evoked excitatory responses. These GABA responses were dependent on extracellular chloride, and antagonised by picrotoxin, but not by bicuculline suggesting an ionotropic receptor distinct from $GABA_A$ receptors (Nistri and Sivilotti, 1985; Sivilotti and Nistri, 1989). Similarly, Arakawa and Okada (1988) demonstrated that at low concentrations GABA and the $GABA_A$ receptor agonist muscimol enhanced the amplitude of postsynaptic field potentials (PSP) in slices of guinea-pig superior colliculus, the mammalian equivalent of the frog tectum. At higher concentrations of GABA or muscimol the PSP amplitude was inhibited. The high-affinity GABA responses were insensitive to bicuculline.

Binding studies of [^{3}H]baclofen and [^{3}H]GABA further confirmed the existence of bicuculline- and baclofen-insensitive GABA and CACA binding sites on rat cerebellar and cerebral cortical membranes and in the catfish brain (Drew *et al.*, 1984; Balcar *et al.*, 1986; Myers and Tunnicliff, 1988; Drew and Johnston, 1992). These findings together with studies on receptors with novel pharmacology led to the proposal of a new GABA receptor class termed $GABA_C$ (Drew *et al.*, 1984; Johnston, 1986).

In 1991, Miledi's research group injected *Xenopus laevis* oocytes with poly(A)$^+$ RNA extracted from bovine retina. In addition to GABA responses mediated by $GABA_A$ receptors, they recognised GABA currents mediated by bicuculline- and baclofen-insensitive $GABA_C$ receptors that showed no modulation by barbiturates or benzodiazepines (Polenzani *et al.*, 1991). During the following years they published extensive pharmacological studies on $GABA_C$ receptors (Woodward *et al.*, 1992a; 1992b; 1993) concluding that the $GABA_C$ receptors are more sensitive to GABA than $GABA_A$ receptors, are activated and deactivated more slowly, do not desensitize during GABA application, and are insensitive to $GABA_A$ modulators. In addition, the response to several $GABA_A$ and $GABA_B$ ligands was significantly different in these receptors.

Since then, $GABA_C$ receptors have been reported in several vertebrate retinal

preparations with features similar to those described by Woodward and others (1992a; 1993). Receptors with $GABA_C$-like pharmacology has been shown in the horizontal cells of white perch and catfish (Qian and Dowling, 1993; 1994; Jung *et al*., 1999), as well as in bipolar cells of fish (Dong *et al.*, 1994; Matthews *et al.*, 1994; Qian and Dowling, 1995), salamander (Lukasiewicz and Werblin, 1994; Lukasiewicz *et al.*, 1994), rat (Feigenspan *et al.*, 1993; Pan and Lipton, 1995; Yeh *et al.*, 1996) and ferret (Lukasiewicz and Wong, 1997).

Bipolar cells in organotypic rat retinal slices display GABA-induced inward currents from which 45 ± 16 % are insensitive to bicuculline (Feigenspan and Bormann, 1994). Since a part of the GABA response can be blocked by bicuculline, it is very likely that these cells contain conventional $GABA_A$ as well as $GABA_C$ receptors. In contrast to bipolar cells, the GABA response in amacrine cells of rat retina can be completely blocked by bicuculline (Feigenspan *et al.*, 1993) indicating that these cells have $GABA_A$ but not $GABA_C$ receptors. The conductance and gating properties of the putative $GABA_C$ receptors in the rat bipolar cells are clearly distinct from those of $GABA_A$ receptors, although both have been shown to gate Cl^- selective channels. The GABA response of the $GABA_C$ receptors shows no desensitization and slow recovery after GABA application (Feigenspan *et al.*, 1993). The $GABA_C$ receptors of the rat retina had a 7-fold higher binding affinity for GABA than $GABA_A$ receptors (EC_{50} value being 4.2 μM and 27.1 μM, respectively), and the saturating current for $GABA_C$ receptors was only 260 pA in contrast to 1500 pA shown for $GABA_A$ receptors (Feigenspan and Bormann, 1994). The single channel conductance of the $GABA_C$ receptors was 7-8 pS in contrast to 27-29 pS obtained for $GABA_A$ receptors in the same cell (Feigenspan *et al.*, 1993; Feigenspan and Bormann, 1994). The Hill coefficient for both receptors was close to 2, and the pore diameter was similar in both receptors in an open state (Feigenspan and Bormann, 1994).

In the horizontal cells of the white perch (Qian and Dowling, 1993; 1994) and the bipolar cells of the tiger salamander (Lukasiewicz *et al*., 1994) and the goldfish (Matthews *et al*., 1994), GABA-activated currents are fully resistant to inhibition by bicuculline suggesting that in retina of the lower vertebrates, the $GABA_A$ and $GABA_C$ receptors are not expressed in the same cells. The putative $GABA_C$ receptors of the fish and salamander retinae, however, share the properties described above for $GABA_C$ receptors of the rat retina.

1.2.2.2. Pharmacology of $GABA_C$ receptors

The GABA molecule exhibits conformational flexibility because the bonds within the molecule can rotate freely (Johnston, 1968) and different conformations of GABA have been shown to interact with different types of GABA receptors (Andrews and Johnston, 1979). GABA seems to activate the $GABA_C$ receptors in a partially folded conformation (Johnston, 1992). This conformation is possible also for muscimol, TACA (*trans*-aminocrotonic acid), and CACA (*cis*-aminocrotonic acid). $GABA_A$ re-

ceptors are activated by GABA in a more extended conformation, which is not accessible by CACA. CACA has been shown to stimulate possible $GABA_C$ receptors in cat spinal interneurons (Johnston *et al.*, 1975), rat, bovine, salamander and fish retina (Feigenspan *et al.*, 1993; Woodward *et al.*, 1993, Qian and Dowling, 1993; Matthews *et al.*, 1994; Lukasiewicz *et al.*, 1995; Pan and Lipton, 1995), frog optic tectum (Sivilotti and Nistri, 1989), and rat superior colliculus (Pasternack *et al.*, 1999a;b). As CACA seems to be far less active at $GABA_A$ receptors, it serves as the best selective $GABA_C$ agonist. TACA is equally potent as GABA on $GABA_A$ and $GABA_C$ receptors (Johnston *et al.*, 1975; Sivilotti and Nistri, 1989). Muscimol acts as a potent agonist on both receptor types and isoguvacine, a $GABA_A$ receptor agonist as a partial agonist on $GABA_C$ receptors (Arakawa and Okada, 1988; Woodward *et al.*, 1993; Pasternack *et al.*, 1999a;b).

Bicuculline, a competitive antagonist of $GABA_A$ receptors, does not have any effect on $GABA_C$ receptors. Apparently, the agonist binding pocket of $GABA_C$ receptors does not allow binding of bicuculline providing an excellent method to distinguish GABA-responses mediated by $GABA_A$ and $GABA_C$ receptors.

$GABA_A$ receptor channel blocker picrotoxin (PiTX) inhibits $GABA_C$ receptors in amphibian and fish retina (Lukasiewicz and Werblin, 1994; Zhang and Slaughter, 1995; Qian *et al.*, 1997; Dong and Werblin, 1998), as well as in ferret retina (Lukasiewicz and Wong, 1997). As has been shown for $GABA_A$ receptors (Newland and Cull-Candy, 1992), the action of PiTX on $GABA_C$ receptors is use-dependent, as PiTX interacts with open Cl^- channels (Dong and Werblin, 1996). In contrast to other preparations studied, the responses of the $GABA_C$ receptors in the rat retinal neurons are rather resistant to PiTX (Feigenspan *et al.*, 1993; Pan and Lipton, 1995; Nelson *et al.*, 1999) indicating that differences in amino acid structure or receptor stoichiometry may change the channel pore of $GABA_C$ receptors (see also chapters 1.2.2.4.2. and 5.2.3.).

In 1996, Ragozzino and co-workers (Ragozzino *et al.*, 1996) reported the design of a selective $GABA_C$ receptor antagonist, TPMPA [(1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid]. Pasternack and others (1999a;b) used this compound to identify $GABA_C$ receptors in the rat superior colliculus slices. When only low concentrations of GABA, muscimol or CACA were used, TPMPA completely inhibited their effects.

Imidazole-4-acetic acid (I4AA), a $GABA_A$ receptor agonist, has been reported to reduce or block responses mediated by $GABA_C$ receptors in rat, perch and teleost retina (Qian and Dowling, 1994; 1995; Pan and Lipton, 1995; Qian *et al.*, 1997). On $GABA_C$ receptors of the salamander retinal bipolar cells, however, I4AA seems to act as a partial agonist (Lukasiewicz and Shields, 1998).

$GABA_C$ receptors are insensitive to many $GABA_A$ receptor modulators, such as diazepam, flunitrazepam and pentobarbital (Feigenspan *et al.*, 1993; Qian and Dowling, 1993; Lukasiewicz *et al.*, 1994).

Some partial agonists of $GABA_A$ receptor, such as THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol), P-4-S (piperidine-4-sulfonic acid), 3-APS (3-aminopropanesulfonic acid), ZAPA (Z-3-[amidinothio]propenonic acid), $GABA_A$ receptor antagonist SR-95531, $GABA_B$ receptor agonists 3-APA (3-aminopropylphosphonic acid), 3-APMPA (3-aminopropyl[methyl]phosphinic acid), a $GABA_B$ antagonist DAVA (δ-aminovaleric acid), and a glycine receptor antagonist strychnine were all shown to have antagonistic effects on bovine retinal $GABA_C$ receptors (Woodward *et al.*, 1993).

1.2.2.3. ρ subunits form $GABA_C$ receptors

In an attempt to clone the cystic fibrosis chloride channel, Cutting and collaborators (1991) cloned a new GABA subunit from human retina cDNA library by PCR, using oligonucleotides designed for highly conserved regions of $GABA_A$ receptor subunits (α, β, γ and δ). This new subunit was found to share 30-38% similarity on the amino acid level to known $GABA_A$ receptor subunits, a level observed previously between subunit classes. It was therefore suggested to belong to a subunit class of its own, and was named ρ_1, according to its site of origin, retina (Cutting *et al.*, 1991). Soon, a second ρ subunit, ρ_2, was cloned from human retina by the same group (Cutting *et al.*, 1992). The ρ_2 subunit was 74% homologous to ρ_1 on the amino acid level, which is comparable to the degree of conservation between $GABA_A$ receptor subunits within one class (α_{1-6}, β_{1-4}, γ_{1-3}). To date, the ρ_1 and ρ_2 subunits have been cloned from human, rat, mouse, chicken, and perch retinas (Cutting *et al.*, 1991; 1992; Albrecht and Darlison, 1995; Ogurusu *et al.*, 1995; **I**; Qian *et al.*, 1997; 1998; Greka *et al.*, 1998; see also Table 1 in chapter 5.1.1.). A third member of the ρ subunit class, ρ_3 subunit, has been cloned from rat and perch (Ogurusu *et al.*, 1996; Qian *et al.*, 1998). From white perch retina, additional subtypes of ρ_1 (ρ_{1A} and ρ_{1B}) and ρ_2 (ρ_{2A} and ρ_{2B}) subunits have been cloned and sequenced (Qian *et al.*, 1998). In addition, a splice variant of human ρ_1 with a 51 nucleotide deletion in the putative extracellular domain has been cloned (Martinéz-Torres *et al.*, 1998). Our studies also suggest the existence of possible spliced variants for ρ_2 (**II**) and ρ_3 (unpublished observation) subunits in the rat brain. The similar primary structure of the ρ and $GABA_A$ receptors subunits implies similar protein folding (Cutting *et al.*, 1991) and possible pentameric assembly for $GABA_C$ receptors (Fig. 1). The existence of different isoforms and spliced variants of ρ subunits suggest the existence of several different $GABA_C$ receptors that can be formed by homooligomeric or heterooligomeric assembly of different ρ subunits.

Closer examinination of the molecular similarity reveals that ρ subunits are closely related to both $GABA_A$ and glycine receptor subunits. $GABA_A$ receptor subunits β and δ are the closest relatives to ρ subunits with 37-45% similarity on the amino acid level. Similarity of ρ subunits with other $GABA_A$ receptor and glycine receptor subunits is 30-35 %. The ρ clones from white perch retina have similar in-

tron-exon boundaries as those of other ionotropic GABA receptor genes, suggesting that the ρ gene and other GABA receptor genes are derived from a common ancestor (Qian *et al.*, 1997). Genetic mapping of $GABA_A$ receptor subunit genes localizes them on human chromosomes 4, 5, 15, and the X, where they are found tightly clustered (Bailey *et al.*, 1999a). These four clusters of $GABA_A$ receptor subunit genes have been hypothesized to have arisen by duplication of a progenitor cluster containing α-like, β-like, and γ-like subunit genes (Greger *et al.*, 1995; McLean *et al.*, 1995). The ρ_1 and ρ_2 subunit genes are mapped to human chromosome 6q14-q21 suggesting that they have arisen from a duplication of a ρ progenitor (Cutting *et al.*, 1992). ρ_3 subunit gene is mapped to human chromosome 3q11-q13.3 (Bailey *et al.*, 1999b). Since none of the ρ subunit genes is localized with the $GABA_A$ receptor gene clusters, Bailey and others (1999b) suggest that the $GABA_A$ and $GABA_C$ receptor subunit genes diverged at an early stage in the evolution of the ionotropic GABA receptor family.

$GABA_A$, $GABA_C$ and glycine receptors belong to the superfamily of inhibitory ligand-gated ion channels (ILGIC). Based on maximum-parsimony and distance matrix methods comparing the protein sequences of receptor subunits, the ρ, β and δ subunits of GABA receptors have been suggested to form a phylogenetic branch of their own in the ILGIC (Xue, 1998). Unlike other GABA-mediated subunits in the ILGIC, ρ, β and δ subunits are not involved in benzodiazepine binding. Furthermore, the phylogenetic branch of ρ subunits could be considered closest to the archetype of the GABA receptors, since many invertebrate GABA receptor subunits that are thought to be evolutionary intermediates rather than well-differentiated $GABA_A$ receptor subunits are classified in this branch (Fig. 2). In agreement with this hypothesis, many

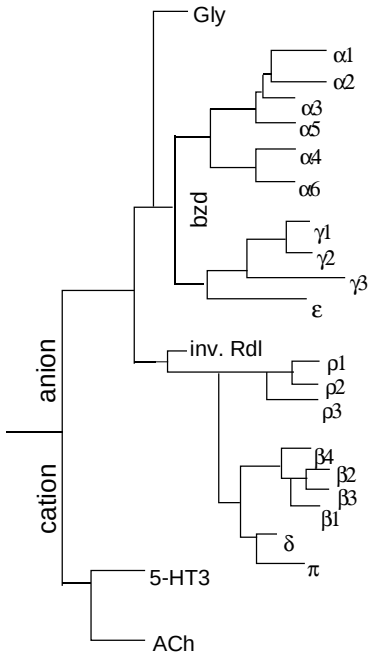

Fig. 2. A dendrogram illustrating the relations between the known $GABA_A$ and $GABA_C$ receptor subunits, as well as invertebrate GABA receptor Rdl subunit (inv. Rdl). Adapted from Ortells and Lunt, (1995), Hosie et al. (1997), Xue (1998) and Barnard et al. (1998).

pharmacological properties of crayfish GABA receptors are similar to those of $GABA_C$ receptors (for review, see Kaila, 1994).

In addition to the evidence provided by *in vitro* expression studies showing similar kinetic and pharmacological properties between $GABA_C$ receptors and receptors formed of ρ subunits, results from single cell RT-PCR in combination with patch-clamp recordings (Yeh *et al*., 1996), and *in situ* hybridization (Enz *et al*., 1995; Qian *et al*., 1997) and immunocytochemistry (Enz *et al*., 1996; Koulen *et al*., 1997; 1998; Wässle *et al*., 1998a) that localize ρ subunits to cells with $GABA_C$-like pharmacology indicate that ρ subunits are the basic components of the $GABA_C$ receptors.

1.2.2.4. Functional characteristics of receptors formed of ρ subunits

1.2.2.4.1. Receptors formed of ρ_1 subunits

When human ρ_1 mRNA was injected into *Xenopus* oocytes, it was translated into functional subunits that formed homooligomeric receptor channels responsive to GABA with characteristics similar to $GABA_C$ receptors. ρ_1 receptors showed high affinity for GABA (EC_{50} 1-5μM; Shimada *et al*., 1992; Kusama *et al*., 1993b; Enz and Bormann, 1995; Wang *et al*., 1995b), insensitivity to $GABA_A$ receptor antagonists bicuculline, SR-95531, securinine and (+)-tubocurarine (Shimada *et al*., 1992; Kusama *et al*., 1993b), $GABA_A$ receptor modulators such as benzodiazepines and barbiturates (Shimada *et al*., 1992; Calvo *et al*., 1994) and $GABA_B$ receptor agonists baclofen and faclofen (Shimada *et al*., 1992).

Activation by CACA has also been recorded on transiently expressed human ρ_1 receptors (Kusama *et al.*, 1993b; Calvo *et al*., 1994). Muscimol and TACA act as a potent agonists, and isoguvacine and I4AA act as partial agonists on homooligomeric ρ_1 receptors (Shimada *et al.*, 1992; Kusama *et al.*, 1993b; Calvo *et al*., 1994; Pan and Lipton, 1995; Wegelius *et al.*, 1997; Chebib *et al.*, 1998). In addition, TAMP (*trans*-2-aminomethyl-cyclopropylcarboxylic acid) and CAMP (*cis*-2-aminomethyl-cyclopropylcarboxylic acid) (Kusama *et al.*, 1993b), and in the C2 position substituted analogues of GABA and TACA (Chebib *et al*., 1997) have been shown to have agonistic effects on human ρ_1 receptors.

Human ρ_1 receptors are highly sensitive to inhibition by picrotoxin (PiTX) and t-butylbicyclophophoro-thionate (TBPS) that are $GABA_A$ receptor channel blockers (Cutting *et al*., 1991; Shimada *et al.*, 1992; Kusama *et al*., 1993b; Enz and Bormann, 1995; Wang *et al*., 1995b). PiTX inhibition of human ρ_1 receptors expressed in *Xenopus* oocytes is 30 times weaker and TBPS inhibition 250 times weaker than that of $GABA_A$ receptors (Shimada *et al.*, 1992). 2-MeTACA has been shown to be an antagonist in human ρ_1 receptors (Chebib *et al.*, 1998).

The pore diameter of homooligomeric human ρ_1 receptors was estimated to be 0.61 nm, which is slightly larger than the 0.51 nm estimated for the $GABA_C$ receptors of the rat retina (Feigenspan and Bormann, 1994), or the 0.56 nm of the $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Wotring *et al*., 1999). The single channel chord conductance of

human ρ_1 receptors was 0.65 pS when the internal Cl^- concentration was 20mM (Wotring *et al*., 1999).

1.2.2.4.2. Receptors formed of ρ_2 subunits

Human ρ_1 and ρ_2 subunits share 83% amino acid sequence homology, and their GABA affinity and binding co-operativity are very similar when expressed alone (Wang *et al*., 1994; 1995b), which is in agreement with the fact that the proposed GABA binding site in ρ_1 subunit is completely conserved in ρ_2 subunit (Amin and Weiss, 1994). Human ρ_2 receptors are slightly more sensitive to most agonists than ρ_1 receptors (Kusama *et al*., 1993a; Enz and Cutting, 1999a). The human ρ_1 and ρ_2 receptors are equally insensitive to $GABA_A$ receptor modulators including bicuculline, hexobarbital, and diazepam. However, actions of some GABA agonists/antagonists are different on ρ_1 and ρ_2 receptors. The sensitivity of human ρ_1 receptors to the $GABA_C$ receptor antagonist TPMPA is 8-fold higher than that of ρ_2 receptors (Chebib *et al*., 1998). In addition, 2-MeTACA has been found to be a competitive antagonist at ρ_1 receptors, but a partial agonist at ρ_2 receptors (Chebib *et al*., 1998). The differences in agonist/antagonist binding could be due to differences in conformational flexibility of the ligands and in folding of the subunits. Information obtained from these results could be used to differentiate between possible native ρ_1 and ρ_2 receptors.

Homooligomeric human ρ_2 receptors are more sensitive to PiTX than ρ_1 receptors (Wang *et al*., 1994). The PiTX sensitivity of homooligomeric rat ρ_1 receptors is similar to human ρ_1 receptors (Wang *et al*.; 1995b; Zhang *et al*., 1995; Enz and Cutting, 1999a; **I**), but interestingly, homooligomeric mouse ρ_2 receptors (Greka *et al*., 1998) as well as native $GABA_C$ receptors of the rat retina are found to be rather insensitive to PiTX (Feigenspan *et al*., 1993; Pan and Lipton, 1995). Zhang and others (1995) co-expressed rat ρ_2 and ρ_1 subunits and discovered PiTX-insensitive GABA-induced receptors similar to rat $GABA_C$ receptors. Mutation studies indicated that a methionine residue of rat ρ_2 subunit in the second transmembrane region (TM2) of the subunit, in place of threonine which is conserved in rat and human ρ_1, human ρ_2 and rat ρ_3 subunits, is responsible for PiTX-resistance of receptors formed by co-expression of rat ρ_1 and ρ_2 subunits (Zhang *et al*., 1995). Therefore, this methionine residue of ρ_2 subunits is also likely to cause the PiTX resistance of native rat $GABA_C$ receptors, suggesting that $GABA_C$ receptors in the rat retina are heterooligomeric rather than homooligomeric.

Despite the high similarity between ρ_1 and ρ_2 subunits, the GABA-induced whole-cell currents of human ρ_1 receptors give significantly bigger amplitudes than currents recorded from human ρ_2 receptors (Kusama *et al*., 1993a; Wang *et al*., 1994; Hackam *et al*., 1997a; Enz and Cutting, 1999a). Low current amplitudes seem to be a universal feature of homooligomeric ρ_2 receptors of different species. GABA-induced amplitudes of homooligomeric mouse (Greka *et al*., 1998) and white perch (Qian *et al*., 1997) ρ_2 receptors are comparable to currents seen in human ρ_2 receptors. The

sequences responsible for the difference in current amplitudes of ρ receptors were shown to reside in the N-terminus of the subunit prior to the first transmembrane region within a region of 100 amino acids, which may be involved in N-glycosylation or subunit assembly of the ρ_1 receptor (Hackam *et al.*, 1997b; Enz and Cutting, 1999a). It is possible that the formation and/or stability of ρ_1 receptors are better than of ρ_2 receptors and that N-glycosylation causes longer half-life or more stable secondary structure for ρ_1 receptors.

The rat ρ_2 subunits have not been shown to form functional receptor channels on their own when expressed in *Xenopus* oocytes (Zhang *et al.*, 1995). Different expression techniques, e.g. intranuclear injection of ρ_2 cDNA rather than ρ_2 cRNA (Qian *et al.*, 1997) or changing the expression vector (Kusama *et al*., 1993a) affect the amount of expression of ρ_2 receptors and the responses obtained from them. In addition, 20 times higher concentrations of ρ_2 than ρ_1 RNA have been used when injecting *Xenopus* oocytes (Wang *et al.*, 1994). It is possible that the stability of the mRNA or cRNA used or the absence of proteins needed for multimerization or targeting to cell membrane affect the transfection efficiency. For human ρ_2 receptors, more efficient expression was observed by Enz and Cutting (1999a) when HEK-293 cells rather than *Xenopus* oocytes were used. Still, the current amplitudes of ρ_2 receptors were about 4 times smaller than those observed for ρ_1 receptors when equal amounts of cDNA were used for transfections. Since human and mouse ρ_2 subunits are capable of forming functional homooligomeric channels it is likely that rat ρ_2 subunits will form functional channels when expressed *in vitro* in mammalian cell lines.

1.2.2.4.3. Receptors formed of ρ_3 subunits

So far, ρ_3 subunits have been cloned only from rat (Shingai *et al.*, 1996) and perch (Qian *et al*., 1998). The GABA-induced current amplitudes of homooligomeric rat ρ_3 receptors expressed in *Xenopus* oocytes were significantly lower than those observed from homooligomeric rat and human ρ_1 receptors, and even somewhat smaller than currents seen from homooligomeric human ρ_2 receptors. In addition, the affinity for GABA (EC_{50} 7.5 μM; Shingai *et al*., 1996) was lower than that of homooligomeric human and rat ρ_1, or human ρ_2 receptors. The GABA-response of ρ_3 receptors was insensitive to bicuculline, but antagonized by picrotoxin. Pentobarbital and diazepam reduced the GABA-induced current slightly, but neurosteroid was ineffective on ρ_3 receptors (Shingai *et al*., 1996). Our preliminary findings on HEK-293 cells transfected with rat ρ_3 cDNA suggest that these mammalian cells do not express homooligomeric ρ_3 receptors on their cell membrane (Fig. 3; Hirvelä *et al.*, 1999). In agreement with this, the ρ_3 subunits cloned from white perch retina did not form functional homooligomeric receptors when expressed in *Xenopus* oocytes (Qian *et al*., 1998). It is possible that in the rat, ρ_3 subunits combine with ρ_2 to form heterooligomeric receptors as indicated by recent recordings from cells expressing ρ_2 and ρ_3 subunits (Ogurusu *et al*., 1999).

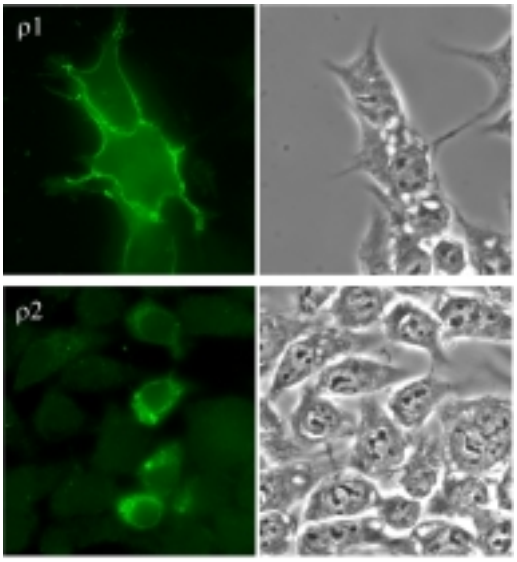

Fig. 3. Confocal microscopy pictures showing HEK-293 cells transfected with ρ_1 or ρ_3 subunit cDNA, and stained with ρ antibody. ρ_1 subunits are expressed in the cell membrane, but it appears that ρ_3 subunits are not.

1.2.2.5. Regulation of ρ receptors by intracellular factors

Currents mediated by $GABA_C$ or ρ_1 receptors have been shown to be regulated by Ca^{2+}, phosphatases and kinases (Feigenspan and Bormann, 1994; Kaneda *et al*., 1997; Kusama *et al*., 1995). However, the six consensus phosphorylation sites of the ρ_1 subunit (Cutting *et al*., 1991; **I**) are not phosphorylated directly by kinases (Kusama *et al*., 1998; Filippova *et al*., 1999), but receptor internalization via interaction with the cytoskeleton has been suggested to underlie the phosphorylation-dependent regulation of the ρ_1 receptors (Filippova *et al*., 1999).

Microtubule-associated protein 1B (MAP-1B) has recently been found to link ρ_1 subunits to the cytoskeleton at bovine retinal synapses (Hanley *et al*., 1998). MAP-1B was demonstrated to interact with the large intracellular domain of ρ_1 subunits, colocalize with them at bipolar cell axon terminals and cause their redistribution when co-expressed in COS cells. The same did not apply to ρ_2 subunits, suggesting that these subunits may either assemble with ρ_1 subunits and form heterooligomeric receptors or form homooligomeric receptors in the cell membrane through a different mechanism.

Homooligomeric ρ_1 receptors have also been shown to interact with the glycine transporter GLYT-1E/F (Hanley *et al*, 2000). This interaction may be important for modulating the responses of the $GABA_C$ receptors by changes in the concentration of glycine.

1.2.2.6. Assembly properties of ρ subunits

ρ and $GABA_A$ receptor subunits have a high degree of amino acid sequence similarity in regions believed to be responsible for oligomerization (Cutting *et al.*, 1991; Hackam *et al.*, 1997a; 1997b), but most evidence point to the conclusion that ρ subunits do not co-assemble with $GABA_A$ receptor subunits. α_1, α_5 and β_1 subunits failed to co-immunoprecipitate with ρ_1 or ρ_2 subunits (Hackam *et al.*, 1998) and coexpression of ρ subunits with α and β subunits produced GABA receptors with identical properties to those obtained from homooligomeric ρ receptors (Shimada *et al.*, 1992). In the retina, ρ and $GABA_A$ receptor subunits have been localized to the same cell type (Yeh *et al.*, 1996; Enz *et al.*, 1996), but antibody stainings localize ρ, $GABA_A$ and glycine receptor clusters in separate synapses (Wässle *et al.*, 1998a; Koulen *et al.*, 1998) supporting further the idea that ρ subunits do not co-assemble with $GABA_A$ or glycine receptor subunits in the mammalian brain.

Results from studies on the possible heterooligomerization of the mammalian ρ subunits indicate that the native $GABA_C$ receptors are either homooligomeric or heterooligomeric receptors formed exclusively by ρ subunits. The N-terminus has been found to be responsible for the assembly of the ρ subunits (Hackam *et al.*, 1997a; 1997b; 1998; Enz and Cutting, 1999a). Recently, a 70 amino acid N-terminal region has been identified in the human ρ_1 subunit to contain sequences for the homooligomeric assembly of the ρ_1 subunits (Enz and Cutting, 1999b). It is possible that another domain in the ρ_1 subunit recognizes the ρ_2 or ρ_3 subunit, since several domains have been shown to be responsible for subunit specific assembly in the glycine receptor (Kuhse *et al.*, 1993). Co-transfection of human ρ_1 and ρ_2 subunits generated whole-cell currents with large amplitudes characteristic of ρ_1 receptors, but more rapid inactivation typical for receptors formed of ρ_2 subunits (Enz and Cutting, 1999a). This result implies that human ρ_1 and ρ_2 subunits readily form heterooligomeric receptors. However, some differences in assembly efficiency was found indicating that for human ρ receptors, homooligomerization might still be more efficient than heterooligomerization and that both homo- and heterooligomeric receptors are likely to form *in vivo* (Enz and Cutting, 1999a).

In addition to human, the rat and mouse ρ_2 subunits have been shown to form heterooligomeric receptors with ρ_1 subunits (Zhang *et al.*, 1995; Greka *et al.*, 1998; Enz and Cutting, 1999a) and with ρ_3 subunits (Ogurusu *et al.*, 1999) with kinetic and pharmacological properties of the native $GABA_C$ receptors found in the rat retina (Feigenspan *et al.*, 1993). In agreement with these results, ρ_1 and ρ_2 subunit transcripts have been found in the bipolar cells (Yeh *et al.*, 1996), while ρ_2 and ρ_3 subunit transcripts have been found in the ganglion cells of rat retina (Yeh *et al.*, 1996; Ogurusu *et al.*, 1997), suggesting that different $GABA_C$ receptor types are expressed in these cells.

From the white perch retina, five clones of ρ subunits have been found (ρ_{1A}, ρ_{1B}, ρ_{2A}, ρ_{2B}, ρ_3; Qian *et al.*, 1997; 1998), suggesting the existence of several different

$GABA_C$ receptors in the perch retina that can be formed by homooligomeric or heterooligomeric assembly of different ρ subunits. All perch ρ_1 and ρ_2 subunits formed homooligomeric receptors that showed unique kinetic and pharmacological properties, but none of them were similar to responses obtained from native perch $GABA_C$ receptors (Qian *et al.*, 1997; 1998). Surprisingly, one of the perch ρ subunits, ρ_{1B}, was shown to co-assemble with human $GABA_A$ receptor γ_2 subunit (Qian and Ripps, 1999) and to form $\rho_{1B}\gamma_2$ receptors with many characteristics similar to native perch GABA receptors found from perch retina (Qian and Dowling, 1993; 1995; Qian *et al.*, 1997a; 1997b). Therefore, it is possible that some bicuculline-insensitive ionotropic GABA receptors of the perch retina are formed by heterooligomerization of ρ_{1B} and γ_2 subunits.

Mammalian ρ_1 subunits are more homologous to perch ρ_{1A} subunit that does not form heterooligomeric receptors with $GABA_A$ receptor subunits than to ρ_{1B} subunit, indicating that coassembly between mammalian ρ_1 subunits with γ_2 subunit is likely not to happen. Comparison of the N-terminal regions of the mammalian and perch ρ_1 subunits shows that the 70 aa region responsible for homooligomerization of ρ_1 subunits (Enz and Cutting, 1999b) is very conserved in all ρ_1 subunits (e.g. 92% between rat and perch ρ_1 subunits). This further stands for the assumption that the signal for heterooligomerization of the ρ subunits can be found in some other domain of the ρ subunit sequence.

1.2.2.7. Conclusions on the properties of $GABA_C$ vs. $GABA_A$ receptors

The question of whether ρ subunits should be classified into $GABA_A$ receptors or a separate $GABA_C$ receptor class is still under debate (e.g. Barnard *et al.*, 1998).

The $GABA_A$ and $GABA_C$ receptors share basic functional characteristics of ionotropic receptors activated by the same transmitter. In both receptors, GABA and muscimol induce anion conductance that is blocked by picrotoxin. However, other pharmacological and kinetic properties of $GABA_C$ receptors distinguish them from classical $GABA_A$ receptor responses. Especially important differences between these two receptors are their sensitivities to agonists/antagonists suggesting that the binding pockets in these receptors are different. The concentrations of GABA, muscimol and CACA that cause half-maximal activation of the $GABA_C$ receptors are too low to cause detectable responses in the $GABA_A$ receptors. On the other hand, bicuculline and TPMPA are highly selective antagonists to only one of these GABA receptors. The GABA-mediated responses of the $GABA_C$ receptors are insensitive to the typical modulators of the $GABA_A$ receptor channels, such as benzodiazepines and barbiturates indicating that also the regulatory binding sites in these receptors are very distinctive. In addition, the marked differences in activation and deactivation kinetics, as well as in desensitization during GABA application, differentiate $GABA_A$ and $GABA_C$ receptors functionally.

Of course, the pharmacological picture of GABA receptors in the nervous system in general is not that simple. Different $GABA_A$ receptor subunit combinations have been reported to be responsible for differences in their sensitivities to ligands of the GABA receptors both *in vivo* and *in vitro* (for review, see Barnard *et al.*, 1998). However, for vertebrate preparations, the insensitivity to bicuculline can be considered as a quite reliable tool for distinguishing between different ionotropic GABA receptors. In the retina, where these two ionotropic GABA systems have been localized to the same cell, it is reasonable to separate them from each other with a different name.

Several other molecular and functional observations favor the separation of vertebrate $GABA_A$ and $GABA_C$ receptors. In contrast to $GABA_A$ receptors that need different subunits (at least α and β) to form functional channels, the ρ subunits form functional homooligomeric GABA-gated channels with similar properties to native $GABA_C$ receptors found in retina. Even though some mammalian β subunits have been shown to be able to form homooligomeric channels with small currents (Blair *et al.*, 1988; Sigel *et al.*, 1989; Krishek *et al.*, 1996), these channels are often open in the absence of GABA, and similar native $GABA_A$ receptors have not been demonstrated so far.

In addition, growing knowledge on the functional characteristics of the $GABA_C$ receptors in the retina (Feigenspan and Bormann, 1994; Wellis and Werblin, 1995; Dong and Werblin, 1998; Lukasiewicz and Shields, 1998; Roska *et al.*, 1998) and elsewhere in the nervous system (Nistri and Sivilotti, 1985; Arakawa and Okada, 1988; Munakata *et al.*, 1998; Platt and Withington, 1998; Pasternack *et al.*, 1999a;b; **IV**) indicate a specific role for these receptors separate from $GABA_A$ receptors.

In conclusion, there is molecular and functional evidence to classify $GABA_C$ receptors separately from $GABA_A$ receptors, but in the nomenclature A-B-C, it would be reasonable that the close relatives $GABA_A$ and $GABA_C$ receptors were next to each other, and not separated by the metabotropic $GABA_B$ receptor. Other terms like $GABA_A\rho$ (Shimada *et al.*, 1992), $GABA_{NANB}$ (Martina *et al.*, 1995) or $GABA_{A0r}$ (Barnard *et al.*, 1998) have been suggested, but we see no reason not to use the $GABA_A$/$GABA_C$ classification already in wide use in the scientific literature.

1.2.3. IONOTROPIC GABA RECEPTORS IN INVERTEBRATES

In invertebrates, both bicuculline-sensitive and -insensitive GABA receptors have been found. It is interesting to note that some crustacean ionotropic GABA receptors have been found to share several pharmacological properties including bicuculline-insensitivity, as well as the absence of prominent desensitization that are typical for $GABA_C$ receptors (Takeuchi and Takeuchi, 1967; 1969; Takeuchi and Onodera, 1972). However, GABA receptors of crayfish leg opener muscle show a

classical $GABA_A$ receptor agonist profile (Fig. 4; Wegelius *et al.*, 1997). In this preparation, GABA and muscimol were more potent agonists than THIP and TACA, while I4AA and CACA evoked only 10-15 % of current amplitudes induced by GABA. Similar to $GABA_C$ receptors, the GABA-responses of the crayfish GABA receptors are not modulated by diazepam (Wegelius *et al.*, 1997). In the lobster thoracic ganglia neurons that show GABA responses with $GABA_C$-like pharmacology (Jackel *et al.*, 1994) the measured single-channel conductance was about 8 times higher than in the bicuculline-resistant GABA receptors of the rat retina.

The GABA receptors of the fruit fly (*Drosophila melanogaster*) are insensitive (ffrench-Constant *et al.*, 1993; Chen *et al.*, 1994), but those of pond snail (*Lymnea stagnalis*) sensitive to the blocking effect of bicuculline (Harvey *et al.*, 1991). The bicuculline-resistant GABA-receptors of fruit fly seem to be composed of Rdl (resistance to dieldrin) subunits that readily form homooligomeric receptors (ffrench-Constant *et al.*, 1993; Chen *et al.*, 1994). Interestingly, the Rdl subunit of *Drosophila melanogaster* displays 43 % homology on the amino acid level to rat ρ_1 subunit, while other cloned GABA receptor subunits from fruit fly show closer similarity to other $GABA_A$ receptor subunits (Hosie *et al.*, 1997). Immunocytochemical studies have shown products of the Rdl gene distributed throughout the central nervous system of D. *melanogaster*, but especially concentrated e.g. in the optic lobes and optic system of the brain (Hosie *et al.*, 1997). Therefore, it seems that the Rdl subunits are the insect counterparts of vertebrate ρ subunits (Fig. 2; Hosie *et al.*, 1997).

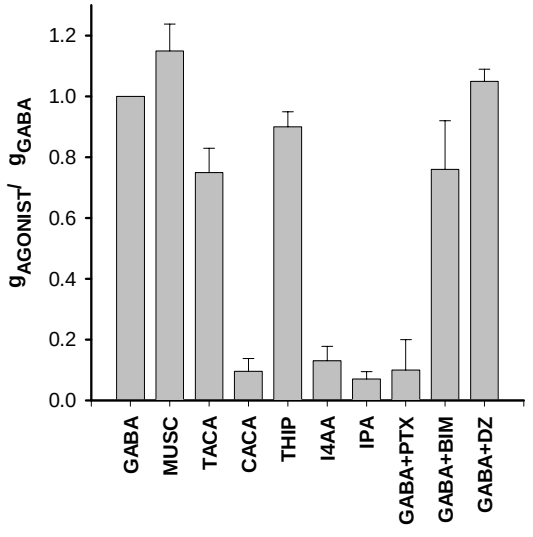

Fig. 4. GABA receptors of crayfish muscle fibers are insensitive to bicuculline- and benzodiazepines, but show $GABA_A$-like agonist pharmacology (Wegelius et al., 1997). Abbreviations: MUSC, muscimol; TACA, trans-aminocrotonic acid; CACA, cis-aminocrotonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol; I4AA, imidazole-4-acetic-acid; IPA, isonipecotic acid; PTX, picrotoxin; BIM, bicuculline, DZ, diazepam.

Rdl subunits of fruit fly form homooligomeric GABA-activated Cl^- channels highly sensitive to picrotoxin, but insensitive to bicuculline and benzodiazepine, when expressed in *Xenopus* oocytes (Chen *et al.*, 1994). However, invertebrate bicuculline-resistant GABA receptors are distinguished from vertebrate $GABA_C$ receptors by their sensitivity to modulation by some $GABA_A$ receptor modulators such as benzodiazepines, pentobarbital and neurosteroids (Chen *et al.*, 1994, Hosie and Sattelle, 1996). In addition, the high potency and efficacy of isoguvacine and ZAPA distinguishes invertebrate GABA receptors from the $GABA_C$ receptors, but the insensitivity to bicuculline and the low potency of SR-95531, a $GABA_A$ receptor antagonists, distinguishes them from the $GABA_A$ receptors (Hosie and Sattelle, 1996).

It is evident that the invertebrate GABA receptors do not fit into the vertebrate categories of $GABA_A$ and $GABA_C$ receptors. Possibly the inhibitory mechanism of invertebrate nervous system is simpler and does not require different ionotropic GABA receptors systems.

1.3. METABOTROPIC GABA RECEPTORS

1.3.1. $GABA_B$ RECEPTORS

In contrast to the ionotropic nature of $GABA_A$ and $GABA_C$ receptors, the metabotropic $GABA_B$ receptors are coupled to cation channels via GTP-binding proteins. This indirect and slower response to GABA by $GABA_B$ receptors compared to ionotropic GABA receptors causes either an increase in the cell's permeability to K^+ or a decrease in Ca^{2+} permeability (Bowery, 1989). The stimulation of postsynaptic $GABA_B$ receptors causes a prolonged neuronal hyperpolarization. In addition, $GABA_B$ receptors are present on presynaptic terminals, where they function as autoreceptors suppressing the release of neurotransmitters. The $GABA_B$ receptors are selectively activated by baclofen and inhibited by phaclofen, but are not affected by bicuculline (Bowery *et al.*, 1980; Hill and Bowery, 1981; Bowery, 1989). Other agonists used for characterization of the $GABA_B$ receptors are 3-aminopropyl phosphinic acid and its methyl homolog AMPPA (SKF97541), (RS)-4-amino-3-(5-chloro-2-thienyl)butanoic acid (BCTG) and 3-aminopropylphosphonic acid (3-APPA). In addition, 2-hydroxysaclofen, CGP35348 and CGP36742 have been used as selective antagonists for the $GABA_B$ receptor. However, no agonist that would differentiate between putative $GABA_B$ receptor subtypes has been found to date (Bowery and Enna, 1999).

Two different subclasses of the $GABA_B$ receptor, GBR1 (of which two isoforms GBR1a and GBR1b are known) and GBR2 have been cloned (Kaupmann *et al.*, 1997) with seven membrane-spanning domains in each subunit. Recently, $GABA_B$ receptors have been shown to exist as heterodimers of GBR1 and GBR2 (Kaupmann *et al.*, 1998; Möhler and Fritschy, 1999). The $GABA_B$ receptors are widely distributed throughout the mammalian brain and spinal cord suggesting that they play an important role in the inhibitory system of the CNS (Hill and Bowery, 1981). Results pre-

senting data on synapses in rat neocortical and hippocampal slices where the GABA-responses were solely mediated by either $GABA_A$ or $GABA_B$ receptors (Thomson and Destexhe, 1999) bring new ideas for determining the role of $GABA_B$ receptors in the nervous system.

2. AIMS OF THE STUDY

The results presenting ionotropic GABA receptors with novel pharmacology and the cloning of ρ subunits from human retina opened an interesting new field of GABA receptor research. These novel GABA receptor subunits were shown to be highly localized to retina and to form homooligomeric receptors *in vitro*. Some reports suggesting the existence of $GABA_C$ receptors beside $GABA_A$ receptors in the mammalian brain prompted us to look closer to this possibility. The quest to find specific functional roles for two separate GABAergic ionotropic systems in the nervous system is intriguing. Our aims in the studies included in this thesis were

- To clone and to identify ρ subunits in the rat central nervous system.
- To study the distribution and co-localization of the ρ subunit transcripts in the postnatal and adult rat nervous system.
- To characterize the pharmacology and pH sensitivity of rat ρ_1 homooligomeric receptors expressed *in vitro*.

3. MATERIALS AND METHODS

Animals (II, IV)

Wistar rats of ages P0 (postnatal day 0), P8, P14, P21, and adult (P60) were decapitated and relevant tissues were dissected, frozen and stored in –70°C until used (**II, IV**). For immunocytochemistry and electrophysiological recordings in **IV**, young adult pigmented Long Evans or Wistar rats were used. Deeply anaesthetized animals were either decapitated or perfused through heart, and the brain was cut into slices as described in **IV**.

RNA isolation (II, IV)

Total RNA was extracted from frozen tissue by the method of Chomczynski and Sacchi (1987; **II**) or with SV Total RNA isolation system (Promega; **IV**).

Cell cultures and transfection (I, III)

Human embryonic kidney cells (HEK-293) were cultured in appropriate conditions described in publication **III**. Cells were transfected with ρ_1 cDNA using calcium phosphate precipitation technique by Chen and Okayama (1987).

Cloning of rat ρ_1 subunit (I)

The full-length rat ρ_1 subunit was cloned by using rat retinal cDNA library in lZAP (Stratagene) donated by Dr. Nakanishi. The ρ clones were identified with a ^{32P}-labelled 700 bp PCR fragment of ρ_2 subunit.

In situ hybridization (II, IV)

Sagittal, coronal or horizontal cryosections of rat brain were stored at –70°C until used. Sections were fixed in 4% PFA, hybridized using radiolabeled ρ cRNA sense or antisense probes and appropriate SP6 or T7 transcription system (Promega), and performed as described in detail in publication **II**.

Southern and Northern analysis (II)

The detection of the amplified cDNA samples from RT-PCR (Southern) or ρ subunit mRNAs from different rat brain tissues (Northern) was done by blotting electrophoretically separated cDNA or total RNA to Hybond-N filters (Amersham) and by hybridizing them with radiolabeled ρ cDNA probes.

RT-PCR (II, IV)

1 mg of total RNA was reverse-transcribed using random hexamer primers, dNTP and Supercript II™ reverse transcriptase (Life Technologies; **II, IV**). The conditions and primers used in the standard (**II**) and semiquantitative (**IV**) PCR experiments are described in relevant publications.

Immunocytochemistry (IV)

The antiserum generated against the N-terminus of the rat ρ_1 subunit recognizes all known ρ subunits (ρ_1, ρ_2 and ρ_3; Enz *et al.*, 1996) and was used to study the distribution of receptors formed of ρ subunits in the brain of adult rat. The conditions used for the immunostaining of coronal 30 mM thick cryosections are described in detail in **IV**.

Whole cell patch clamp recordings (I, III)

Membrane currents were recorded in the whole cell patch clamp mode from transfected HEK-293 cells with an EPC-9 patch clamp (HEKA) as described in publications **I** and **III**.

Field potential recordings (IV)

Coronal 450 mM thick brain sections were used for recording extracellular population excitatory postsynaptic potentials (pEPSPs) in the CA1 region of the hippocampus. A bipolar stimulation electrode was placed in the stratum radiatum layer and a recording electrode in the stratum pyramidale layer. $GABA_C$ agonist and antagonist were used to selectively activate or inhibit the $GABA_C$ receptors.

4. RESULTS

4.1. CLONING OF THE RAT ρ_1 SUBUNIT (I)

To find homologues of ρ subunits from a species that could be widely used for several lines of experimental work (e.g. distribution and functional characterization studies), we cloned the full-length ρ_1 subunit transcript from rat retinal cDNA library (**I**), and a 700 bp partial ρ_2 sequence from retinal cDNA template (**I**). The ρ_1 cDNA consisted of 4182 nucleotides with an open reading frame of 1422 base pairs (bp) corresponding to 474 amino acids. The rat ρ_1 sequence also included a 5´ 29 bp and 2.6 kilo base (kb) 3´ untranslated region with a sequence homologous to MER18 repeat.

Based on a hydrophobicity plot, the amino acid (aa) sequence of ρ_1 subunits consists of 4 putative transmembrane regions (TM1-TM4) with 6 putative consensus sequence sites for phosphorylation by protein kinase C (PKC; Kusama *et al.*, 1995), a 260 aa long N-terminal extracellular region, a 100 aa long intracellular loop between TM3 and TM4, and a 2 aa long extracellular C-terminal end. The ρ_1 cDNA sequence was published in EMBL database (accession number X95579, **I**).

4.2. EXPRESSION OF ρ SUBUNIT TRANSCRIPTS IN THE RAT NERVOUS SYSTEM

We studied by Northern analysis, *in situ* hybridization, and RT-PCR the expression of ρ_1, ρ_2 and ρ_3 subunit mRNAs in the rat nervous system (**II**, **IV**).

4.2.1. Expression detected by Northern blot (II)

Northern analysis with total RNA revealed ρ_2 transcript in the superior colliculus, hippocampus and cortex. A weak band could also be seen in the cerebellum, but not in the olfactory bulb or the brain stem (**II**). Our probe for ρ_2 subunit recognized three different-sized transcripts, of which 2.3 and 2.0 kb mRNAs were found in the retina, hippocampus and superior colliculus, but only 2.0 kb in the cortex.

ρ_1 transcripts could only be seen in the retina, and the size of the ρ_1 mRNA was 4.2 kb (**II**), which is in agreement with the size of our full-length ρ_1 cDNA cloned from rat retinal cDNA library (**I**).

4.2.2. Expression detected by *in situ* hybridization (II, IV)

With *in situ* hybridization, we detected high expression of ρ_2 transcript in the inner nuclear layer of the retina within bipolar cells (**II**). Expression in this layer could be seen in the rat eye from postnatal day 8 (P8) to adulthood (P60; **IV**). At P0, the retinal layering was incomplete, and no clear labeling with ρ_2 probe could be seen (**IV**).

Expression of ρ_2 but not ρ_1 or ρ_3 transcript could be detected in the adult rat brain outside retina (**II, IV**). The strongest expression of ρ_2 mRNA was found in the superficial gray layer (SGL) of the superior colliculus (SuC). We also found expression of ρ_2 transcript in the CA1 region of the hippocampus, as well as in several regions of the rat visual system, including the dorsal lateral geniculate nucleus (LGNd; **II**), cortex (**II; IV**), the pretectal nucleus of the optic tract (NOT; **IV**) and the medial terminal nucleus of the accessory optic system (MTN; **IV**).

ρ_2 mRNA expression was visible in SuC already at P0 (**IV**). The signal in the SuC increased during development and was strongest in adulthood (P60). In the CA1 region of the hippocampus (HC) the expression could be detected at P8 (**IV**). The expression of ρ_2 mRNA in the CA1 region was maximal at P14 but prominent still in adulthood. In the NOT and in the MTN, expression of ρ_2 mRNA could be seen from P14 to P60 (**IV**). The cortical expression of ρ_2 mRNA was detectable only in the adult rat (P60; **IV**).

4.2.3. Expression detected by RT-PCR (IV)

We used a semi-quantitative RT-PCR method with 18S rRNA as an endogenous control to study the relative distribution of the ρ subunits in the nervous system of postnatal rat.

Expression of ρ_1 mRNA was seen in the retina, SuC, HC, DRG and spinal cord at P0 and in adult rat. Similarly, expression of ρ_3 mRNA was detected in these regions, except in adult SuC. In general, expression of ρ_2 mRNA was more pronounced than expression of ρ_1 or ρ_3 transcripts in adult tissues. At P0, expression of ρ_1 or ρ_3 transcripts was less abundant than ρ_2 mRNA in the retina, and SuC. In hippocampus, the expression levels of ρ_1 and ρ_3 transcripts were relatively high compared to ρ_2 at P0, and in adult, the expression of ρ_3 mRNA was equally high as ρ_2 mRNA.

By comparing to results obtained by *in situ* hybridization of ρ_2 subunit, it appears that the expression of ρ_1 and ρ_3 subunits is also up-regulated during postnatal development in the retina, but down-regulated in the hippocampus.

4.2.4. Expression detected by immunocytochemistry (IV)

Strong punctate labeling was detected throughout the SGL of the SuC, and weaker labeling was also seen in the optic layer. In addition, immunopositive labeling was detected in the LGN, the NOT, and the AOS nuclei, where the heaviest density of immunopositive puncta was found in the DTN, but strong immunoreactivity was found also in the MTN and the LTN.

4.3. TRANSIENTLY EXPRESSED RAT ρ_1 SUBUNITS FORM FUNCTIONAL HOMOOLIGOMERIC RECEPTORS SIMILAR TO $GABA_C$ RECEPTORS (I, III)

The full-length (4.2 kb) ρ_1 subunit cDNA was transiently expressed in human

embryonic kidney cells (HEK-293). Using patch clamp, we were able to measure GABA currents that were activated slowly and reversed at the reversal potential for Cl^- ions. These GABA currents were inhibited by 100 μM picrotoxin, but were insensitive to 100 μM bicuculline (**I**, **III**). The concentration-response experiments revealed an EC_{50} of 3.0 ±0.05 μM for GABA and a Hill coefficient of 1.8 ±0.07 (n=3) (**III**).

4.4. PROTONS MODULATE ρ_1 RECEPTOR FUNCTION (I, III)

To find out whether endogenous ionic modulators of GABA receptors, H^+ and Zn^{2+}, have an effect on $GABA_C$ receptors, we studied the GABA responses of the rat and human ρ_1 receptors at various extracellular pH levels (pH_o 5.5. to 9.0) and in the presence of extracellular Zn^{2+} (**I, III**). The GABA-induced Cl^- conductance mediated by rat ρ_1 receptors decreased by extracellular protons throughout the pH_o range studied. The GABA current measured at pH 8.4 was 140 % and at pH 6.4 50 % of that observed at pH value 7.4 (**I**). Changes in GABA-induced (3 μM) conductance in different extracellular pH levels were associated to two protonation sites on rat ρ_1 receptors with pKa 6.4 and pKa 8.2 (**III**). The affinity of H^+ for the site with low pKa decreased with increasing GABA concentration while the site with high pKa showed only a slight dependence on GABA concentration (**III**). However, the human ρ_1 receptors under similar conditions showed no sensitivity to pH_o>7.4 suggesting that human ρ_1 subunits display only the non-competitive inhibition of H^+ associated with low pKa (**III**).

A chimeric receptor, with extracellular (*N*-terminal) amino acids from the rat ρ_1 cDNA and transmembrane and intracellular (carboxy-terminal) amino acids from the human ρ_1 receptor, displayed proton sensitivity similar to that observed for the rat receptor (**III**).

4.5. ZINC INHIBITS ρ_1 RECEPTORS (III)

10 μM Zn^{2+} inhibited the GABA-induced conductance of the ρ_1 receptors. The inhibition mediated by Zn^{2+} was competitive, since the affinity of the ρ_1 receptors for GABA was decreased by Zn^{2+}. The inhibitory effect of Zn^{2+} decreased with decreasing pH_o, and turned to enhancement below pH 6.4.

4.6. $GABA_C$ RECEPTORS IN THE HIPPOCAMPUS (IV)

Low concentrations of CACA (0.75 and 1.5 μM) reduced the population spike amplitude of the hippocampal CA1 pyramidal neurons. 1 μM TPMPA inhibited the effect of 3 μM CACA on the population spike. CACA (5 μM) was also shown to reduce the hyperexcitability induced by 5 μM bicuculline in the CA1 region.

5. DISCUSSION

5.1. ρ_1 SUBUNITS CLONED FROM RAT RETINA FORM FUNCTIONAL HOMOOLIGOMERIC RECEPTORS SENSITIVE TO CHANGES IN EXTRACELLULAR pH

5.1.1. Rat ρ_1 subunit is highly homologous to human ρ_1 subunit

ρ_1 subunit was first cloned from human retina by Cutting and others (1991). We cloned the rat counterpart of ρ_1 subunit that is 95 % homologous to the human ρ_1 subunit on the amino acid level. Simultaneously with us, Zhang and co-workers cloned the rat ρ_1 cDNA sequence with a putative amino acid sequence identical to ours (Zhang *et al.*, 1995).

Subunit	%	Reference
Rat ρ_2	75	Ogurusu *et al*., 1995
Rat ρ_3	62	Ogurusu *et al*., 1995
Human ρ_1	95	Cutting *et al*., 1991
Mouse ρ_1	98	Greka *et al*., 1998
Chicken ρ_1	87	Albrecht and Darlison, 1995
Perch ρ_{1A}	74	Qian *et al*., 1998
Perch ρ_{1B}	70	Qian *et al*., 1998

Table 1. % homology on the amino acid level of ρ_1 subunits from different species to rat ρ_1 subunit

5.1.2. Rat ρ_1 subunits form functional receptors similar to native $GABA_C$ receptors

Using the whole cell patch clamp, we recorded GABA currents from human embryonic kidney cells (HEK-293) transiently expressing rat ρ_1 subunit cDNA (**I**, **III**). The GABA currents were bicuculline-insensitive, but sensitive to picrotoxin, and similar to those measured from homooligomeric human ρ_1 subunits expressed in *Xenopus* oocytes (Kusama *et al.*, 1993a; 1993b; Shimada *et al.*, 1992; Amin and Weiss, 1994) or HEK-293 cells (Enz and Cutting, 1999a). Both receptors had similar half-maximal GABA responses (**III**), showed slow activation and deactivation, and no desensitization.

5.1.3. Differences in the pH sensitivity of rat and human ρ_1 receptors

In a study by Wang and others (1995a) no pH sensitivity of GABA-induced currents was observed in human ρ_1 receptors. This is in contrast with our studies of rat and human ρ_1 receptors (**I, III**), and with studies on the pH-sensitivity of murine and rat $GABA_A$ receptors (Krishek *et al.*, 1996; Pasternack *et al.*, 1996).

The GABA-induced currents of rat and human ρ_1 receptors were decreased by an increase in extracellular H^+ concentration (**III**). However, rat ρ_1 receptors were sensitive to protons throughout the pH range studies, but human ρ_1 receptors were insensitive to pH_o>7.4. For rat receptors, two mechanisms of H^+ inhibition associated with two protonation sites with different pKas were observed. The affinity of H^+ for the site with low pKa decreased with increasing GABA concentration while the site with high pKa showed only a slight dependence on GABA concentration. The insensitivity of the human ρ_1 subunits for the changes in pH_o at alkaline levels suggests that the human receptor displays only the non-competitive inhibition of H^+ associated with low pKa.

A chimeric receptor, with extracellular (*N*-terminal) amino acids from the rat ρ_1 cDNA and transmembrane and intracellular (carboxy-terminal) amino acids from the human ρ_1 receptor, displayed proton sensitivity similar to that observed for the rat receptor (**III**). Since the rat and human ρ_1 receptors are highly homologous on the amino acid level of their extracellular domain, this result indicates that the major difference in the pH sensitivities can be attributed to 11 N-terminal amino acids that differ between these subunits. These amino acid differences are grouped in four clusters of 2-3 amino acids (**III**). In the rat ρ_1 receptor, a histidine residue (pKa 6.0, free in solution) is a possible cause for the observed alkaline pH sensitivity. In the human ρ_1 receptor, two glutamate residues with charged side chains are located on each side of this histidine. In the rat receptor one of the glutamates is replaced by an aspartate with similar charge and the other with an uncharged glycine residue. This change in local charge may increase the pKa of histidine and hence introduce the alkaline pH sensitivity.

5.1.4. Modulation of ρ_1 receptors by Zn^{2+} is pH sensitive

Recent studies have shown that, like $GABA_A$ receptors, $GABA_C$ receptors (Dong and Werblin, 1995; 1996; Han and Yang, 1999) and homooligomeric ρ receptors are modulated by Zn^{2+} (Calvo *et al.*, 1994; Chang *et al.*, 1995; Wang *et al.*, 1994; 1995a; **III**). In addition to Zn^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+} inhibited GABA currents of homooligomeric human ρ_1 receptors (Calvo *et al.*, 1994). In retina, Zn^{2+} effectively blocks the $GABA_C$ currents in catfish horizontal cells (Dong and Werblin, 1996) and carp bipolar cells (Han and Yang, 1999). Zn^{2+} has been shown to be concentrated in synaptic terminals of rod and cone photoreceptors (Wu *et al.*, 1993), which make synapses with horizontal cells, where Zn^{2+} may be co-released with transmitters to modulate the activation of native $GABA_C$ receptors (Dong and Werblin, 1995; 1996). In skate bipolar cells, Zn^{2+} strongly enhances GABA currents, but this effect was found to be due to $GABA_A$ receptors, since in the presence of 100 μM bicuculline, Zn^{2+} depressed GABA-evoked currents by almost 50% (Qian *et al.*, 1997). In addition, opposite effects on the kinetics of $GABA_A$ and $GABA_C$ receptors by Zn^{2+} has been demonstrated in carp bipolar cells. Zn^{2+} slows down activation and desensitiza-

tion of the carp $GABA_C$ receptors, but accelerates the response of the $GABA_A$ receptors (Han and Yang, 1999). In addition, deactivation of the $GABA_C$-mediated response is accelerated by Zn^{2+}, but it has no modulatory effect on the deactivation of the $GABA_A$ receptors (Han and Yang, 1999).

It seems that the action of Zn^{2+} is stronger on the native $GABA_C$ receptor than on the ρ receptor (Wang *et al.*, 1994; Dong and Werblin, 1996) possibly due to the heterooligomeric structure of native receptors. In the human homooligomeric ρ_1 receptors, Zn^{2+}-sensitivity has been localized to a histidine amino acid residue in the *N*-terminal domain (Wang *et al.*, 1995a). Studies on $GABA_A$ receptors have shown an effect of pH on the blocking action of Zn^{2+} on GABA-induced Cl^- conductance, suggesting that H^+ and Zn^{2+} might bind to the same site on $GABA_A$ receptors (Smart and Constanti, 1982). This seems to be the case in ρ_1 receptors, since Zn^{2+}-sensitivity of human ρ_1 receptors was found to be dependent on the extracellular pH at acidic levels, decreasing with a decrease in pH (Wang *et al.*, 1995a; **III**). Similarly, the inhibitory effect of Zn^{2+} on GABA-induced currents of rat ρ_1 receptors was sensitive to changes is pH_o (**III**). These findings suggest that the low pKa site common in human and rat ρ_1 subunits could be the histidine residue necessary for Zn^{2+} sensitivity in human ρ_1 receptors.

5.2. ρ SUBUNITS ARE DIFFERENTIALLY EXPRESSED IN DISTINCT AREAS OF THE RAT NERVOUS SYSTEM

5.2.1. ρ subunits in the retina

RT-PCR, *in situ* hybridization, Northern blot analysis and immunohistochemistry have been used to study the expression of ρ subunit mRNA and protein in the nervous system of several species. ρ mRNA expression has been detected in the retina of rat (Enz *et al.*, 1995; Ogurusu *et al.*; 1995; 1997; O'Hara *et al.*, 1995; Albrecht *et al.*, 1997; **II**, **IV**), squirrel (O'Hara *et al.*, 1995); chicken (Albrect and Darlison; 1995; Albrecht *et al.*, 1997) and cow (Cutting *et al.*, 1991; O'Hara *et al.*, 1995). Expression of all known ρ subunit transcripts has been detected by *in situ* hybridization in the rat retina. ρ_1 and ρ_2 mRNAs have been found in the inner nuclear layer (INL) of the rat retina, where bipolar cell somata are situated (Enz *et al.*, 1995; Ogurusu *et al.*, 1995; **II**), and ρ_2 mRNA in chick amacrine and horizontal cells (Albrecht and Darlison, 1995) and perch horizontal cells (Qian *et al.*, 1997). ρ_3 mRNA has been detected only in the rat ganglion cell layer (Ogurusu *et al.*, 1997), where single-cell PCR revealed ρ_2 mRNA as well (Yeh *et al.*, 1996).

In the rat retina, the layering is not completed at the time of birth (postnatal day 0, P0), and no ρ_2 mRNA expression is seen in the migrating cells (**IV**). At P8, ρ_2 mRNA expression can be detected in the INL of the retina (**IV**). This result fits well with immunocytochemical studies of ρ subunits by Koulen and co-workers (1998) showing ρ-immunoreactivity in the rat retinal inner plexiform layer at early postnatal stages (P3), but distinct labeling of bipolar cells only after one week of postnatal

development. Punctate synaptic labeling appeared gradually during the second week of postnatal development (Koulen *et al.*, 1998).

The polyclonal ρ antibody that recognizes ρ_1, ρ_2 and ρ_3 subunits stains $GABA_C$ receptors in the rat, rabbit, macaque monkey (Enz *et al.*, 1996), cat, goldfish, and chicken retina (Koulen *et al.*, 1997). $GABA_C$ receptors were shown to form hot spots at rod and cone bipolar cell axon terminals in the inner plexiform layer of the retina (Koulen *et al*., 1998; Shields *et al*. 2000), whereas $GABA_A$ receptors were found on both ganglion cell processes and bipolar cell terminals (Wässle *et al*., 1998; Shields *et al*., 2000). Electron microscopy showed ρ antibody staining in conventional synapses (in contrast to so called ribbon synapses) that are formed by amacrine cells synapsing to bipolar cells (Wässle *et al.*, 1991; Koulen *et al.*, 1998).

Different morphological classes of bipolar cells in the rat and ferret retina have been demonstrated to contain varying ratios of $GABA_A$ to $GABA_C$ receptors (Euler and Wässle, 1998; Shields *et al*., 2000). The rod bipolar cells have the highest ratio of $GABA_C$ to $GABA_A$ receptors, and the ON cone bipolar cells have a higher ratio of C to A than the OFF bipolar cells (Shields *et al*., 2000). These findings fit well with the differences in the kinetic properties of rod and cone pathways, suggesting that the $GABA_C$ receptors with high affinity to GABA and slow activation and deactivation kinetics are responsible for the slow excitatory responses of the rod bipolar cells (Shields *et al*., 2000). Temporally distinct GABA-responses at the salamander and ferret bipolar cell dendrites and axon terminals indicate that glutamate released from the axon terminals of bipolar cells is inhibited by both $GABA_A$ and $GABA_C$ receptors (Lukasiewicz and Werblin, 1994; Dong and Werblin, 1998; Lukasiewicz and Shields, 1998; Shields *et al*., 2000). A possible role for the different ionotropic GABA receptors is to provide inhibition that is temporally matched with kinetics of glutamate release from bipolar cells (Zhang *et al*., 1997). The retinal ganglion cells are excited at the onset and offset of the light stimulus, and this transient response is caused by a delayed feedback inhibition from amacrine cells to bipolar cell terminals. The feedback inhibition is thought to be mediated through $GABA_C$ receptors that compresses the neural representation both in time (Dong and Werblin, 1998) and in space (Roska *et al*., 2000).

5.2.2. ρ_1 and ρ_3 subunits in the brain

Outside retina, expression of ρ_1 transcript has been shown by Northern analysis in the bovine cerebellum (Cutting *et al*., 1991), by *in situ* hybridization in the rat cerebellar Purkinje layer (Boue-Grabot *et al*., 1998) and cerebellum and optic tectum of 1-day-old chick brain (Albrecht *et al.*, 1997). ρ_3 mRNA has not been detected by these methods outside retina (Ogurusu *et al.*, 1997; **II**). It seems that in the brain, the expression levels of ρ_1 and ρ_3 subunits are markedly lower than in the retina, since many trials to detect them have failed (Ogurusu *et al.*, 1995; 1997; Enz *et al*., 1995; O'Hara *et al.*, 1995; Albrecht *et al.*, 1997, **II**).

By the more sensitive RT-PCR method, it is possible to detect ρ_1 and ρ_3 subunit transcripts in the rat brain (Enz *et al*., 1995; Albrect *et al.*, 1997; Boue-Grabot *et al.*, 1995; 1998; Enz and Cutting, 1999a; **II**, **IV**). With a semi-quantitative RT-PCR method, it became evident that in all regions of the adult rat brain studied, ρ_2 mRNA expression is more abundant than ρ_1 or ρ_3 mRNA expression (**IV**). This in agreement with Enz and Cutting, who detected ratios below 0.5 for ρ_1:ρ_2 by RT-PCR in the thalamus, mesencephalon, cortex and cerebellum of adult rat (Enz and Cutting, 1999a). However, in the hippocampus at the time of birth, ρ_1 and ρ_3 subunits were expressed in excess to ρ_2 mRNA at P0 (**IV**, see also chapter 5.2.5.).

Even though the expression level of ρ_1 and ρ_3 mRNAs is relatively low compared to ρ_2 subunits, the co-localization of mRNA of either one of them in tissues expressing ρ_2 subunits (**IV**), as well as the inefficiency of ρ_2 subunits to form homooligomeric receptors *in vitro* (Zhang *et al*., 1995; Greka *et al*., 1998; Enz and Cutting, 1999a) suggest the existence of heterooligomeric $\rho_2 + \rho_1$ and $\rho_2 + \rho_3$ receptors in the rat brain. In the neonatal hippocampus, coassembly of ρ_1 with ρ_3 subunit is also possible. However, ρ subtype-specific antibody is needed to study the co-localization of ρ subunits in detail.

5.2.3. ρ_2 subunits in the superior colliculus

By far most abundant ρ subunit mRNA expression outside retina has been detected in the superior colliculus (SuC). This brain formation is a multi-layered midbrain nucleus with the highest concentration of GABA and glutamate decarboxylase (GAD, GABA synthesizing enzyme) in the brain, about 50 % of SuC neurons showing GABA-labeling (Mize, 1992). The SuC is part of the mammalian visual system and controls e.g. saccadic eye movements. *In situ* probe designed for ρ_2 labeled the superficial grey layer (SGL) of the rat SuC (**II**, **IV**). This result was confirmed by others in rat brain (Boue-Grabot *et al.*, 1998), as well as in chick optic tectum (Albrecht *et al.*, 1997), the avian homologue to mammalian SuC.

The expression of ρ_2 mRNA in the SGL could be detected already at birth (P0 rat), but the expression level increased during postnatal development being strongest in the adult SuC (**IV**). The strong expression of ρ_2 subunits in the rat SuC (**II**; **IV**; Boue-Grabot *et al.*, 1998; Pasternack *et al.*, 1999b) and the early observations showing bicuculline-insensitive excitatory GABA responses in the guinea-pig SuC (Arakawa and Okada, 1988) and the frog optic tectum (Nistri and Sivilotti, 1985), prompted us to search for a functional role for $GABA_C$ receptors in this region. Electrical stimulation of the optic layer of the SuC slice evoked extracellular field potentials that were recorded in the SGL. The postsynaptic activation decreased by muscimol at concentrations above 10 μM, whereas the opposite effect was observed by concentrations below 1 μM as well as by CACA (Pasternack *et al.*, 1999b). This increase of postsynaptic activation was completely blocked by TPMPA (Pasternack *et al.*, 1999a;b), a competitive antagonist of $GABA_C$ receptors (Ragozzino *et al.*, 1996). These results

suggest that ρ_2 subunits form $GABA_C$ receptors that mediate disinhibition in the rat SuC. The localized presence of both ρ subunit mRNA and protein (**II**, **IV**, Pasternack *et al.*, 1999b) in the SGL indicate that the receptors may be expressed in locally sprouting interneurons. This hypothesis is supported by recent observations of our collaborators, who observed by whole cell patch-clamp almost complete abolishment of the IPSCs by low concentrations of GABA and muscimol in 60% of the SGL neurons (Matthias Schmidt, unpublished observations).

In addition to their role in controlling excitability via disinhibition in the SuC, there are also signs suggesting the involvement of the $GABA_C$ receptors in controlling synaptic plasticity. In the SuC slices, Platt and Withington (1998) have demonstrated that prolonged exogenous application of low concentrations of GABA can cause long term potentiation of field excitatory postsynaptic potential (fEPSP) in SuC. This effect can not be inhibited by bicuculline indicating that the GABA-induced LTP could be mediated by $GABA_C$ receptor activation.

5.2.4. ρ_2 subunits in the visual nuclei and cortex

In addition to the superior colliculus, several other brain regions of the rat visual system showed considerable expression of ρ_2 mRNA. These included the pretectum (NOT; pretectal nucleus of the optic tract; **IV**; Boue-Grabot *et al.*, 1998), dorsal lateral geniculate nucleus (LGNd; **II**), medial terminal nucleus of the accessory optic system (MTN; **IV**), and the visual cortex (**II**, **IV**). ρ subunit immunostaining revealed labeling in all of these subcortical visual nuclei. NOT, MTN and LGNd showed punctate ρ-immunoreactivity that frequently surrounded unstained cell bodies (**IV**). The subcortical visual nuclei that express ρ subunits receive direct input from retina as shown in figure 5. The LGN transmits the signals from the retina to the cortex without much processing, but rather gating them (Daw, 1995). The MTN and NOT are both involved in the generation of optokinetic nystagmus (Simpson *et al.*, 1988) so that horizontally directed image movements are coded by neurons in the NOT, and vertically directed movements by neurons in the MTN. In the visual cortex, objects in and out of the visual context are analyzed in detail by cells that respond either to edges and corners of objects, the color of objects, or the direction of their movement (Daw, 1995).

Several results supporting the functional significance of the ρ subunits in these visual brain regions have been obtained. Electrical stimulation of the MTN has been shown to cause bicuculline-insensitive GABAergic responses in the NOT (Van der Togt and Schmidt, 1994). In addition, bicuculline-insensitive ionotropic GABA receptors have been observed by whole-cell patch clamp in the local interneurons of the LGN (Zhu and Lo, 1999). These results imply that the ρ_2 subunits form functional $GABA_C$ receptors in the brain formations involved in the visual system, and that the $GABA_C$ receptors have a specific role mediating the inhibitory interconnections that control the eye movements in the rat brain.

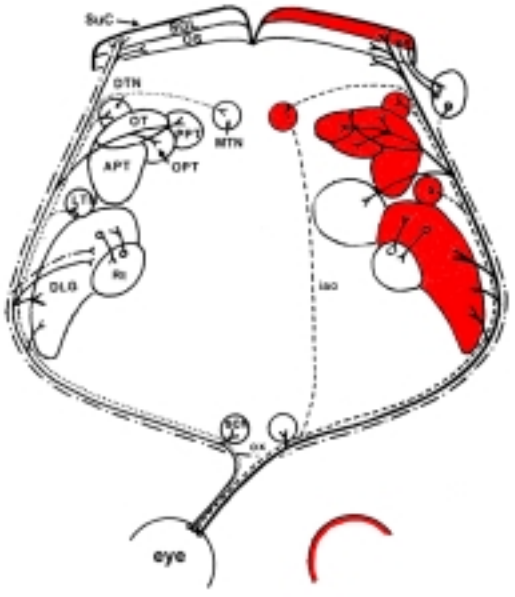

Fig. 5. Representation of the rat visual pathways originating in the retina. Regions marked in red are shown in this thesis to express ρ subunits. Abbreviations: APT, anterior pretectal nucleus; DTN, dorsal terminal nucleus; iao; internal accessory optic tract; LGN, lateral geniculate nucleus; LTN, lateral terminal nucleus; OPT; olivary pretectal nucleus; ox, optic chiasma; PPT, posterior pretectal nucleus, NOT, nucleus of the optic tract; Rt; reticular thalamic nucleus; SCh, suprachiasmatic nucleus; SuC, superior colliculus; SGL, superficial gray layer of the SuC; OS, optic layer of the SuC. (Figure modified from Sefton and Dreher, 1985.)

5.2.5. ρ_2 subunits in the hippocampus

Strong ρ_2 mRNA labeling was detected in the rat hippocampus, where expression was localized to the CA1 region (**II**, **IV**). No expression of ρ_2 transcript was seen at birth (P0), but strong expression was visible from the first postnatal week to the hippocampus of adult rat (**IV**).

During the first postnatal week of rat, depolarizing GABA responses resembling the pharmacology of the $GABA_C$ receptors of the rat and fish retina have been recorded in the hippocampal CA3 pyramidal cells (Cherubini, 1991; Strata and Cherubini, 1994; Martina *et al.*, 1995). This response disappeared during the second postnatal week suggesting that the $GABA_C$ receptors might have a transient role in the developing brain. As no significant expression of ρ_2 could be seen in the hippocampal CA3 region by *in situ* hybridization at any age (**II**, **IV**), it is possible that this subunit has no part in forming the receptors observed by Cherubini and others. By RT-PCR, however, ρ_1 and ρ_3 subunit mRNAs were detected in the hippocampus at P0 in excess to ρ_2 transcript (**IV**). We also observed ρ_1 subunit mRNA in the RNA extracted from dissected CA3 region of young rats (preliminary findings, data not shown). In addi-

tion, expression level of ρ_3 mRNA in the whole brain extract has been shown by RT-PCR to be higher at embryonic day 16 than in the adult rat (Ogurusu *et al.*, 1999). Therefore, it is possible that ρ_1 and ρ_3 subunits compose $GABA_C$ receptors with a transient role in the neonatal rat hippocampus.

In contrast to the transient role in development suggested by Cherubini and others (Cherubini, 1991; Strata and Cherubini, 1994; Martina *et al.*, 1995), the expression of the ρ_2 subunits increases during development (**IV**). By RT-PCR, we could detect expression of ρ_3 mRNA in level comparable to ρ_2 subunit in adult hippocampus. To examine the possible $GABA_C$ receptors formed by ρ subunits in the CA1 region of adult rat hippocampus we electrically stimulated the Schaffer collaterals and recorded the pEPSPs in the CA1 region. We observed a reduction in the population spike of the pyramidal cells by concentrations of CACA that are too low to activate $GABA_A$ or $GABA_B$ receptors. This reduction of the population spike was inhibited by TPMPA indicating that $GABA_C$ receptors are responsible for these responses (**IV**). To further verify this assumption, we tested the effect of CACA during application of bicuculline, and discovered that CACA reduces the hyperexcitability induced by bicuculline (**IV**). These results imply that in the pyramidal cells of the CA1 region of the adult rat hippocampus ρ_2 subunits alone or in combination with ρ_3 subunits form $GABA_C$ receptors that are involved in the inhibitory mechanisms of the hippocampus. ρ subunit-specific immunocytochemistry in the hippocampus is needed to confirm these results and to study the possible expression of $GABA_C$ receptors in the CA3 region.

5.2.6. ρ_2 subunits in other parts of the nervous system

ρ_2 mRNA expression has also been detected by RT-PCR in the rat cerebellum, epithalamus, thalamus, mesencephalon, anterior pituitary, heart and liver (Enz *et al.*, 1995; Enz and Cutting, 1999a; Boue-Grabot *et al.*, 1995; 1998; Albrecht *et al.*, 1997), and recently by *in situ* hybridization in the pars compacta of the substantia nigra (Ogurusu *et al.*, 1999), but no signs of functional $GABA_C$ receptors have been detected in these regions so far.

In the spinal cord, we observed all three ρ subunits by RT-PCR, but failed to detect ρ_2 mRNA by *in situ* hybridization (**IV**). However, ρ subunit-positive immunolabeling has been detected in the rat spinal cord (Wässle *et al.*, 1998b), and TPMPA has been shown to inhibit GABA-responses and rhytmic bursting activity that are possibly mediated by $GABA_C$ receptors in the spinal cord (Rozzo *et al.*, 1999). In addition, a role for $GABA_C$ receptors in mediating nociception in rat spinal cord has been suggested (Louis Stanfa, unpublished observations). It is possible that the putative $GABA_C$ receptors observed in the spinal cord are expressed in the axon terminals of the dorsal root ganglia (DRG) neurons that form synapses in the dorsal horn as indicated by our findings showing relatively high expression of the ρ subunits in the adult DRG (**II**, **IV**). Further studies are needed to understand the structural and

functional properties of receptors composed of ρ subunits in the spinal cord and the DRG.

6. CONCLUSIONS

In this thesis, we presented the sequence of the full-length ρ_1 subunit cloned from the rat retina. The rat ρ_1 subunit was shown to form homooligomeric GABA receptors in a mammalian *in vitro* expression system with properties similar to receptors composed of human ρ_1 subunits and to native $GABA_C$ receptors. In spite of the high homology of the rat and human ρ_1 subunits, an interesting functional difference was seen between the homooligomeric receptors formed of these subunits. Minor differences in their N-terminal amino acid sequence produced differences in their sensitivity to extracellular pH. The high sensitivity of the ρ_1 receptors to pH may contribute to the modulation of the excitation by protons in the rat brain.

The expression of ρ subunits in the retina has been demonstrated in several species. We studied the distribution of all known $GABA_C$ receptor subunits, ρ_1, ρ_2 and ρ_3, in the rat nervous system. The ρ_2 subunit is expressed in excess to ρ_1 and ρ_3 mRNAs in many adult brain regions. It is however possible that the $GABA_C$ receptors in the rat brain are composed of ρ_2 subunits in combination with other ρ_1 or ρ_3 mRNAs subunits. The expression of the ρ_2 subunits in the rat brain increases during development suggesting that they have a functional role in the mature nervous system.

The expression of the ρ_2 subunit shows a distinctive pattern concentrating mainly in the subcortical areas related to movements in the visual field. The reason for such a specific distribution pattern is not known, but it is intriguing that phylogenetically older structures express the GABA receptor archetype. High expression of ρ_2 subunits was also seen in the CA1 region of the hippocampus. In the CA1 region, activation of $GABA_C$ receptors was shown to reduce the population spikes of the pyramidal cells, suggesting that $GABA_C$ receptors have a functionally important role in the general excitability in the rat hippocampus.

In the hippocampus, the $GABA_C$ receptors appear to have an inhibitory role in a similar fashion as shown in the retina, whereas in the superior colliculus and the spinal cord the activation of the putative $GABA_C$ receptors produces a disinhibitory effect (Fig. 6).

The discovery of $GABA_C$ receptor subunit expression in the visual system and in the hippocampus adds a new component to the inhibitory systems in the brain. Future studies will reveal the functional consequences of the highly localized distribution of these receptors.

Inhibitory function			Disinhibitory function	
RETINA	**HIPPOCAMPUS**		**SUPERIOR COLLICULUS**	**SPINAL CORD**
Rod and cone bipolar cells	CA1 pyramidal neurons (Other?)	$GABA_C$ receptor expression	Inhibitory neurons in SGL	Inhibitory neurons in lamina II (Other?)
Amacrine to bipolar cell	?	Synapse	?	DRG neuron to substantia gelatinosa neuron?
Decrease in ganglion cell output	Decrease in CA1 population spike (Decrease in pEPSP?)	Result from receptor activation	Increase in fEPSP	(Decrease in C-fibre and post-discharge in SG neurons) Increase in C-fibre and post-discharge in lamina IV

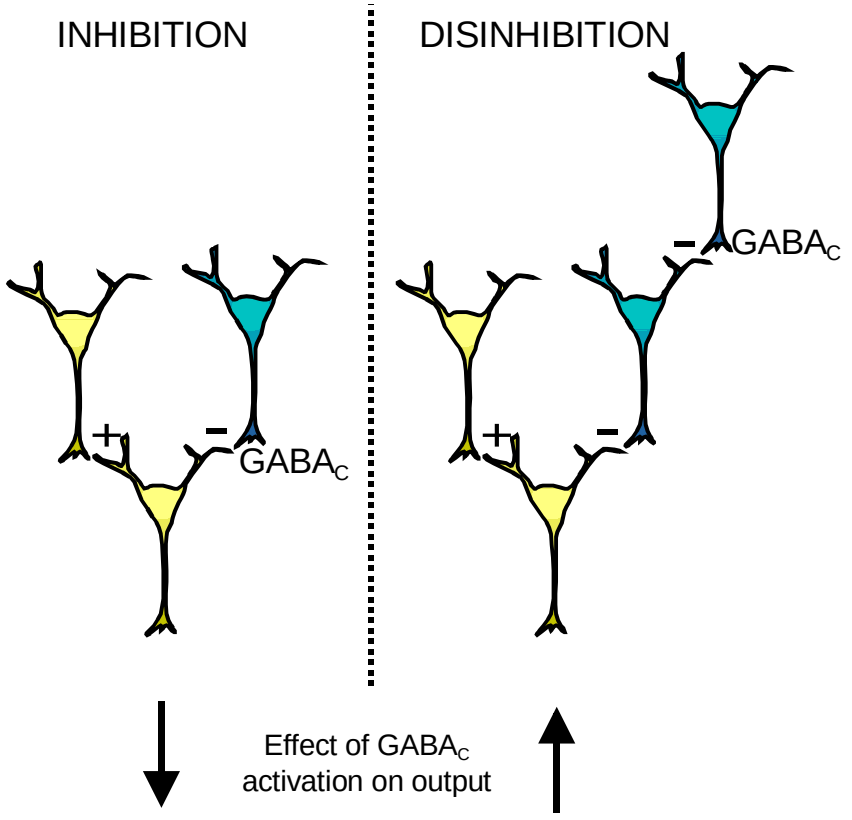

Fig. 6. Possible mechanisms of $GABA_C$ receptor activation in the retina, hippocampus, superior colliculus and spinal cord. In the retina and hippocampus, activation of $GABA_C$ receptors appears to produce direct inhibition while in the superior colliculus and spinal cord a disinhibitory function may occur.

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